

DDR-mediated crosstalk between DNA-damaged cells and their microenvironment

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The DNA damage response (DDR) is an evolutionarily conserved signaling cascade that senses and responds to double-strand DNA breaks by organizing downstream cellular events, ranging from appropriate DNA repair to cell cycle checkpoints. In higher organisms, the DDR prevents neoplastic transformation by directly protecting the information contained in the genome and by regulating cell fate decisions, like apoptosis and senescence, to ensure the removal of severely damaged cells. In addition to these well-studied cell-autonomous effects, emerging evidence now shows that the DDR signaling cascade can also function in a paracrine manner, thus influencing the biology of the surrounding cellular microenvironment. In this context, the DDR plays an emerging role in shaping the damaged tumor microenvironment through the regulation of tissue repair and local immune responses, thereby providing a promising avenue for novel therapeutic interventions. Additionally, while DDR-mediated extracellular signals can convey information to surrounding, undamaged cells, they can also feedback onto DNA-damaged cells to reinforce selected signaling pathways. Overall, these extracellular DDR signals can be subdivided into two time-specific waves: a rapid bystander effect occurring within a few hours of DNA damage; and a late, delayed, senescence-associated secretory phenotype generally requiring multiple days to establish. Here, we highlight and discuss examples of rapid and late DDR-mediated extracellular alarm signals.

Keywords: DNA damage response, senescence, bystander effect, senescence secretome, inflammation, microenvironment, tissue damage

The DNA damage response (DDR) signaling network is essential in the maintenance of genomic stability, via the initiation and coordination of DNA repair mechanisms with appropriate cell cycle arrest checkpoints (d'Adda di Fagagna, 2008; Jackson and Bartek, 2009). The DDR is initially propagated by a series of effective and rapid post-translational modifications culminating in the activation of nodal transcription factors like p53, which organize additional DDR transcriptional responses (Harper and Elledge, 2007).

Briefly, a typical DDR cascade begins with the recruitment and activation of an apical DDR kinase like ATM (ataxia-telangiectasia mutated) to DNA double-strand breaks (DSBs) by damage sensors such as the MRN complex (MRE-11, Rad-51 and NBS-1 proteins). This leads to the local phosphorylation of multiple ATM substrates in the chromatin surrounding the DNA lesion,

almost always including the histone variant H2AX (phospho-H2AX or γ H2AX). These local chromatin modifications provoke the further recruitment of additional DDR mediators at the break, including 53BP1 and MDC1, which amplify chromatin modifications over megabases of DNA generating macroscopic structures called DNA damage foci (DDF; Rogakou et al., 1998; Bonner et al., 2008) that allows for the direct visualization of single DSBs in mammalian cell nuclei (Rogakou et al., 1999). Simultaneously, the distal propagation of the DDR signal within the cell promote cell cycle checkpoints and the activation of p53 (Rodier et al., 2007). When DNA lesions are repairable, the ensuing growth arrest is transient, eventually resulting in cell cycle resumption, and a return to normality. In contrast, severe or irreparable DNA lesions trigger prolonged DDR signaling, resulting in apoptosis or senescence (permanent growth arrest; Campisi and d'Adda di Fagagna, 2007).

The DDR Generates Extracellular Signals

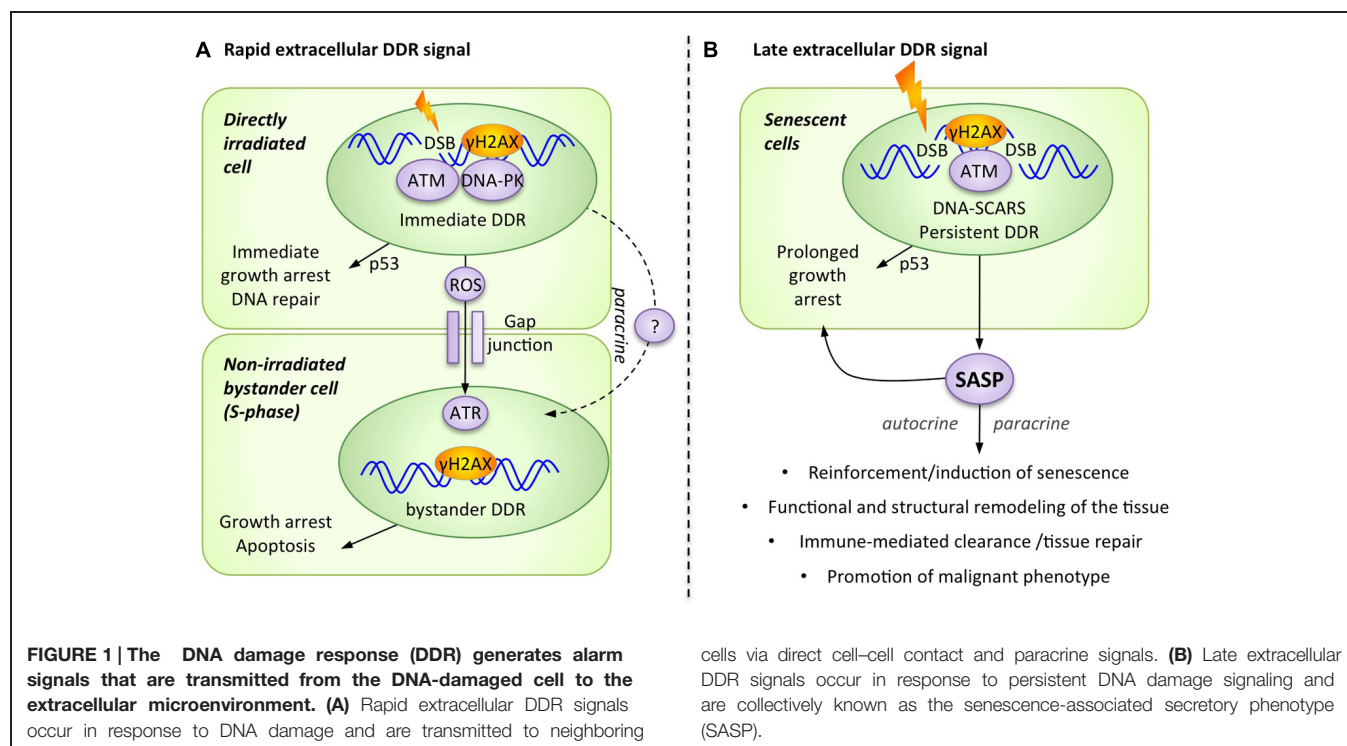
The DDR is mostly known for its role as a cell-autonomous, intracellular signaling cascade that regulates DNA repair and cell cycle checkpoints. However, in the context of higher organisms with multicellular tissues, cells have developed intricate inter-cellular communication mechanisms that the DDR employs to trigger extracellular alarm signals. Conceptually, it is entirely plausible that damaged cells can signal to other cells that their genome has been compromised, essentially generating tissue-wide stress responses. In fact, these DDR-mediated extracellular alarm signals can be subdivided into at least two waves: rapid

and late. While we are still far from a complete understanding of extracellular DDR signaling, it is already well established that specific communication mechanisms including cell surface bound and soluble molecules are involved in this process (Figure 1). Bystander responses received by cells adjacent to damaged cells have been described, and more importantly, some soluble signals have been proposed to travel further in the body, creating additional potential therapeutic intervention opportunities (Tchkonina et al., 2013; Havaki et al., 2014).

A Rapid Extracellular DDR Signal Reaches Undamaged Bystander Cells

Accumulating experimental evidence shows that damaged cells rapidly transmit a DDR-dependent stress signal to neighboring healthy cells, provoking paracrine activation of stress responses such as a bystander DDR. While not originally linked to the DDR itself, this phenomenon was first described under conditions in which only 1% of the cells in a population were irradiated by a low dose of alpha-particles, yet 30% of the cells exhibited chromosomal changes (Nagasawa and Little, 1992). This bystander damage response could be an important mechanism used to rapidly amplify the effect of low dose irradiation by transferring DNA-damage signals from irradiated cells to non-irradiated ones.

It is now clear that non-irradiated cells can adopt common DNA damage-associated phenotypes from adjacent irradiated cells, including micronuclei formation, altered expression of stress-related genes, various epigenetic changes, increased frequency of mutations, induction of apoptosis or senescence, and even malignant transformation (Azzam et al., 2002; Nagasawa and Little, 2002; Morgan, 2003; Ko et al., 2006). Interesting mechanistic evidence supporting the activation of the



cells via direct cell-cell contact and paracrine signals. (B) Late extracellular DDR signals occur in response to persistent DNA damage signaling and are collectively known as the senescence-associated secretory phenotype (SASP).

DDR in bystander cells includes the formation of DNA damage foci (DDF), which also suggests the accumulation of DSBs in these cells (**Figure 1A**; Sokolov et al., 2007). The formation of bystander γ H2AX foci has been observed in a number of experimental systems, including human cultured cells and three-dimensional tissue models, as well as *in vivo* mouse models (Sokolov et al., 2005; Sedelnikova et al., 2007). Furthermore, normal fibroblasts that were exposed to damaged cells, either directly through co-culture or indirectly through conditioned media, demonstrated many other typical DDR markers in DDF, including 53BP1, phospho-ATM, and the focal presence of the ATM-activating MRN complex (Sokolov et al., 2005; Sedelnikova et al., 2007).

The pathways involved in the transmission of alarm signals generated by irradiated cells remain ill defined, but emerging insight appears promising. For example, the activation of DNA-PKcs and ATM is necessary for the generation of a bystander signal from the damaged cell, but these kinases are not required for signal reception in non-irradiated bystander cells (Hagelstrom et al., 2008). Alternatively, the kinase ATR could be required in the recipient bystander cell to allow for the formation of DDF (containing γ H2AX, 53BP1, BRCA1) and the subsequent activation of ATM. Importantly, this ATR-dependent bystander DDR activation occurs only in S-phase cells, consistent with the concept that replication stress is a major trigger for ATR activation (Burdak-Rothkamm et al., 2007, 2008). Accordingly, the radiation-triggered extracellular alarm signal preferentially affects non-irradiated cells that display high rates of replication and transcriptional activities (Dickey et al., 2012). Overall, this suggests that not all bystander cells equally trigger a bystander DDR, and that actively dividing cells are most receptive to this signal (**Figure 1A**).

Two distinct pathways for the transmission of rapid extracellular DDR signals have been proposed: direct cell–cell communication and paracrine interaction (**Figure 1A**). For cells in direct physical contact, small molecules (<1.5 kDa) are usually transmitted through multimeric protein channels termed gap junctions, and the rapid extracellular DDR signal is effectively abrogated following the use of pharmacological inhibitors against gap junctions (i.e., lindane) or by the genetic ablation of an essential gap junction component, connexin 43 (Azzam et al., 1998, 2001). To directly communicate with neighboring cells, the DDR has also been shown to increase the presence of selected cell surface ligands and receptors on damaged cells. For example, some DDR regulated cell surface molecules can subsequently engage surrounding immune cells (NKG2D ligands) or can influence damaged cells survival (DR5 receptor) via receptor-ligand engagement (Wu et al., 1997; Finnberg et al., 2005; Gasser et al., 2005; Lam et al., 2014). A second signaling route consists of the release of soluble factors into the extracellular media, which act in a paracrine manner to stimulate neighboring cells. Consistent with this mechanism, the addition of conditioned media from irradiated cells is sufficient to induce DDF and bystander DDR activation in non-irradiated cells (Sokolov et al., 2005; Shao et al., 2008; Dickey et al., 2009; Klammer et al., 2010).

The molecular players directly tasked with conveying rapid stress signaling from cell to cell are still poorly defined. The

most commonly described family of factors is reactive oxygen or nitrogen species (ROS/NOS), produced at high levels in the damaged cell (Havaki et al., 2014). Indeed, the activation of the DDR as well as its downstream phenotypes in bystander cells (i.e., up-regulation of stress genes, micronucleus formation) is suppressed by superoxide dismutase activation or by ROS inhibitors (Azzam et al., 2002; Little et al., 2002). ROS, and in particular H_2O_2 , which has a relatively longer half-life, can freely diffuse across plasma membranes or through gap junctions, causing DNA damage at distant sites (Azzam et al., 2003). Oxidative stress can result in DNA lesions in the form of single strand DNA breaks (SSBs) that can be converted to DSBs when unresolved or abundant, suggesting that ROS can account for at least a subset of the observed bystander DNA damage events (Tanaka et al., 2006). The second class of soluble factors involved in long distance extracellular DDR signaling includes molecules such as transforming growth factor- β 1 (TGF- β 1) and tumor necrosis factor- α (TNF- α ; Iyer et al., 2000). In addition to its direct role in signaling, the TGF- β 1 secreted by the irradiated cells also contributes to the intracellular increase of ROS and NOS in bystander cells, most likely through NAD(P)H oxidase activation (Burdak-Rothkamm et al., 2007, 2008; Shao et al., 2008). Some, and perhaps most, rapid intercellular damage signaling processes also play a role in the late extracellular response (see below). However, the opposite is not necessarily true, for example, cytokines like IL-6 and IL-8 are exclusive to the late phase following irradiation (Rodier et al., 2009).

A Late Senescence-Associated Extracellular DDR Signal Modifies the Microenvironment

In general, the early phase of the intracellular DDR signaling cascade is a well-established response to nuclear damage, occurring within seconds to hours of the initial assault. But when DNA lesions are particularly severe or irreparable, such as uncapped telomeres (d'Adda di Fagagna et al., 2003), the DDR signal can persist and provoke programmed cell death (apoptosis) or permanent growth arrest (cell senescence; Rodier and Campisi, 2011). While apoptotic cells are rapidly eliminated, damaged senescent cells can persist for extended periods and accumulate in damaged or aging tissues (Baker et al., 2011). Senescence typically depends on the p53/p21 and p16INK4a/RB tumor suppressor pathways (Campisi, 2003; d'Adda di Fagagna, 2008) and is characterized by a series of functional hallmarks (Rodier and Campisi, 2011; Lopez-Otin et al., 2013). It is important to note that the DDR remains permanently activated in most senescent cells, as evidenced by the presence of persistent DDF, termed “DNA segment with chromatin alterations reinforcing senescence” (DNA-SCARS; Rodier et al., 2011). These DNA-SCARS, whether telomeric or intra-chromosomal, are suggested DDR activity nodes that maintain long-term DDR signaling (Rodier et al., 2011).

With few exceptions (Coppe et al., 2011), senescent cells from most species and tissues that are triggered by various stresses all display a Senescence-Associated Secretory Phenotype (SASP; **Figure 1B**), which is critical for the ability of these cells to modulate their microenvironment (Coppe et al., 2008, 2010a,b; Ohanna et al., 2011). A large subset of this SASP critically

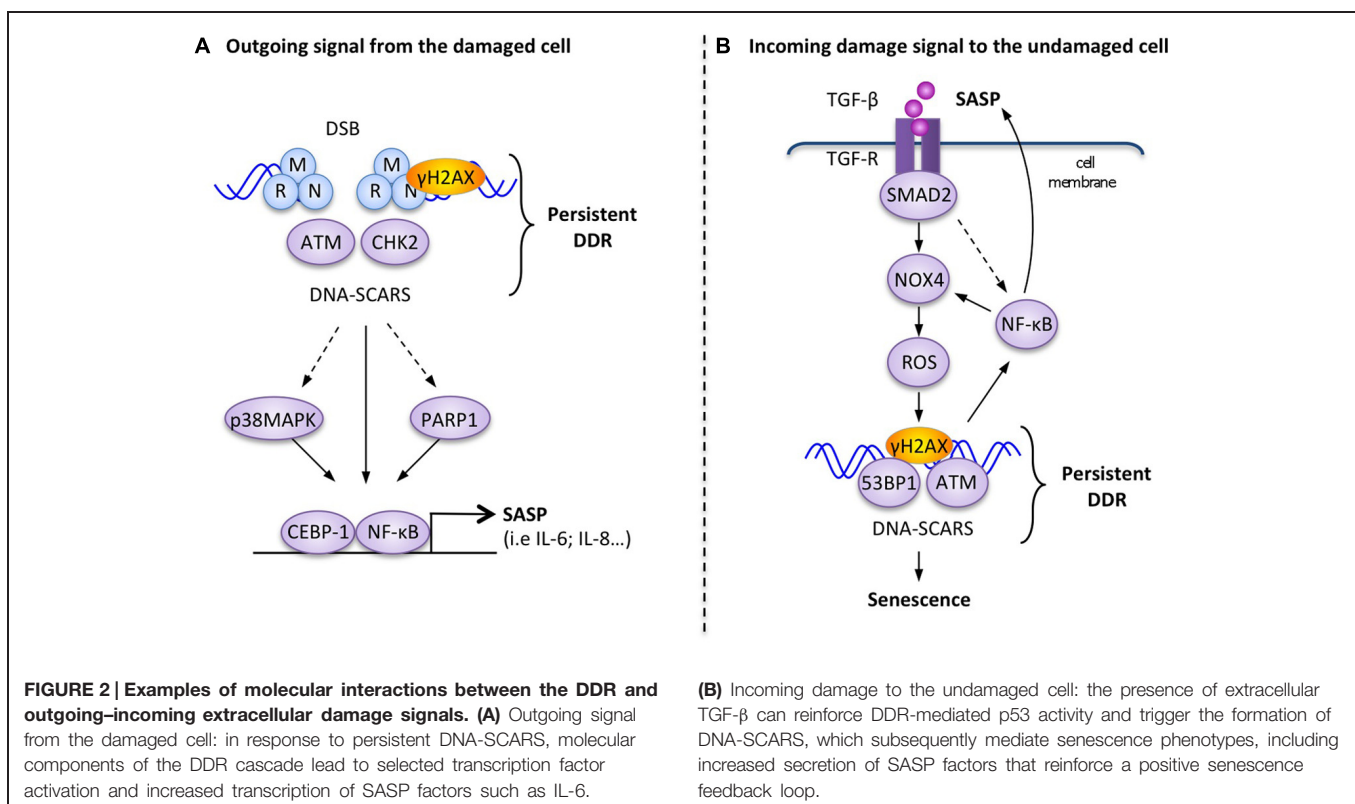
depends on DDR signaling and is thus an extracellular extension of the DDR (Rodier et al., 2009). The SASP is defined as a pro-inflammatory secretome composed of cytokines (i.e., IL-6, IL-8, GRO α , GRO β , MCP-1), growth factors (i.e., GM-CSF, G-CSF, HGF/SF, IGF), proteases (i.e., metalloproteinase MMP-1, -2, and -3), and other non-soluble extracellular matrix proteins (i.e., collagens, fibronectin, laminin; Bavik et al., 2006; Coppe et al., 2008, 2010a; Ohanna et al., 2011; Malaquin et al., 2013). The exact composition of the SASP, its targets, and the overall downstream outcomes vary considerably depending on the cellular context and the type of stresses, but the consensus is that the SASP is at least partially DDR-dependent and is in major part responsible for modulating senescence-associated inflammatory microenvironments in tissues (**Figure 1B**).

The SASP contributes to senescence reinforcement in damaged cells and to tissue repair, but also to age-associated tissue dysfunction and other age-related diseases, including cancer (**Figure 1B**). Because the SASP appears to have both beneficial and deleterious effects, it may represent an interesting, double-edged target for pharmaceutical intervention in human disease (Acosta and Gil, 2012; Perez-Mancera et al., 2014). In the context of cancer, which is particularly applicable to DDR events activated by irradiation or chemotherapy, the SASP also contributes to the clearance of damaged senescent tumor cells by enhancing both innate and adaptive immunity (Xue et al., 2007; Kang et al., 2011; Iannello et al., 2013). However, the SASP also generates chronic inflammation in normal tissues with persistent senescent cells, contributing to age-related tissue dysfunction (Rodier and Campisi, 2011). In the case of

the tumor microenvironment, the SASP of senescent stromal fibroblasts sustains tumor growth and invasion and can even create tumor microenvironments that promote long-term cancer therapy resistance (Krtolica et al., 2001; Sun et al., 2012). Overall, understanding the molecular regulation of the SASP appears essential to reveal how the DDR manages extracellular signaling.

Molecular Regulation of the SASP by the DDR

Direct molecular links between the SASP and the DDR have been demonstrated (**Figure 2A**), but unlike the rapid extracellular DDR signals, the SASP is a slow, delayed response to DDR signaling. While apical DDR kinases like ATM are activated within minutes of DNA lesions and subsequent DDR transcriptional responses are established within hours by p53 and other transcription factors, the SASP develops over days, with associated factors like IL-6 reaching maximal secretion levels 4–10 days after DDR initiation (Coppe et al., 2008; Rodier et al., 2009). In response to DNA damage, persistent DDR signals emanating from DNA-SCARS are necessary, both for the establishment and maintenance of the SASP (Rodier et al., 2009, 2011). At the molecular level, the DDR proteins H2AX, ATM, NBS1 and CHK2, but not cell cycle arrest mediators p53 and pRb, are required to support the SASP (Rodier et al., 2009, 2011). Activation of the p38MAPK stress kinase pathway also triggers the SASP and in some situations concurrent activation of the



DDR is not necessary suggesting that there may be different subsets of SASP factors requiring varying levels of interaction with the DDR (Figure 2A; Freund et al., 2011). For example, the depletion of ATM completely prevents the secretion of IL-6 and IL-8 in senescent irradiated human fibroblasts, but does not impede increased secretion of MCP1, TIMP2, and IGFBP2 (Rodier et al., 2009).

The inflammation-associated transcription factor, nuclear factor- κ B (NF- κ B), is revealing itself to be a master regulator of the SASP (Figure 2A; Salminen et al., 2012). The activation of the RelA p65 subunit of NF- κ B and its recruitment to the chromatin are necessary for the expression of several SASP factors, including IL-6 and IL-8 (Chien et al., 2011). Several studies also showed that the DDR can directly trigger activation of NF- κ B signaling via the interaction between activated ATM and the NEMO protein, which is a regulatory subunit of the IKK complex (inhibitor of NF- κ B signaling). DDR activation results in the export of an ATM/NEMO complex into the cytoplasm, where it binds to and activates IKK α / β , leading to the initiation of NF- κ B signaling via the phosphorylation of inhibitory I κ B proteins (Huang et al., 2003; Wu et al., 2006; Miyamoto, 2011). C/EBP β (CCAAT-enhancer-binding proteins), another transcription factor known to be involved in inflammatory regulation, can also contribute to SASP induction in cooperation with NF- κ B (Kuilman et al., 2008).

Alternatively, another important DNA-damage sensor and DDR regulator, known as PARP-1 (Poly-ADP-ribose polymerase 1), is also involved in the regulation of NF- κ B in senescent melanoma cells undergoing the SASP (Ohanna et al., 2011). Perhaps linked in this context, activated PARP-1 can interact with NEMO to enhance the formation of the ATM/NEMO complex (Stilman et al., 2009).

Cell-Autonomous Reinforcement or Bystander Activation of the DDR Using Late Extracellular DDR Signals (SASP)

Much like the bystander effect described for rapid DDR extracellular signals, the SASP generated from persistently damaged cells is known to modulate DDR-associated behaviors in neighboring cells. Although ROS may influence how the DDR generates the SASP (Guo et al., 2010), most of the SASP's known extracellular effects are currently associated with proteinic soluble factors. Additionally, and again in contrast to the rapid DDR extracellular response, the SASP has been shown to impact both the signal-emitting damaged cell and healthy bystander cells. In damaged cells, the SASP can reinforce p53-associated DDR pathways in a paracrine manner, which maintains senescence in these cells. For example, IL-6 is considered to be a major mediator of paracrine senescence reinforcement (Kuilman et al., 2008). Similarly, CXCR2-binding chemokines (such as IL-8 or GRO-1) are also crucial to reinforce oncogenic- and replication-induced senescence (Acosta et al., 2008). Alternatively, the SASP generated by senescent cells also impacts neighboring bystander cells, as demonstrated both in culture and *in vivo* (Kuilman et al., 2008; Nelson et al., 2012; Acosta et al., 2013). In particular,

multiple SASP components secreted by oncogene-induced senescent cells can trigger paracrine senescence in bystander cells (i.e., TGF β family ligands, VEGF, CCL2, and CCL20) and IL-1 signaling is apparently a major upstream regulator of this paracrine senescence (Acosta et al., 2013). Finally, the SASP factor MCP-1 (CCL2), found in the conditioned media of senescent melanoma cells, was demonstrated to promote DNA lesions in other cells, as illustrated by an increase in 53BP1 DDF (Ohanna et al., 2011). Other extracellular signals that are not necessarily secreted by damaged or senescent cells can also connect to the DDR. For example, type I β -interferon secreted by virally infected cells has been shown to induce paracrine bystander senescence in other cells via the generation of ROS, DDR activation, and p53 activity (Moiseeva et al., 2006).

The link between extracellular signaling and DDR activation is well illustrated by TGF β signaling, which is often associated with senescence (Hubackova et al., 2012; Figure 2B). The inhibition of the TGF β pathway resulted in defective DDR activation in irradiated normal cells, as measured by decreased p53 activation and a reduction in ATM, CHK2, and H2AX phosphorylation (Kirshner et al., 2006). The addition of recombinant TGF β -1 also restored functional ATM in damaged normal cells and could induce DDR-associated senescent phenotypes in healthy hepatocellular carcinoma cells (Kirshner et al., 2006; Senturk et al., 2010). Similarly, TGF β -1 from the conditioned media of senescent normal fibroblasts (oncogene-induced senescent, replicative exhaustion, or genotoxic drugs) triggered a senescent growth arrest in undamaged cells via the DDR-associated p53 or the p16 pathways (Figure 2B). TGF β -induced bystander senescence is associated with the activation of a persistent DDR, the formation of DNA-SCARS, and the subsequent production of SASP factors. It is probable that the activation of the TGF β /SMAD pathway results in increased intracellular ROS and NOS production in the target bystander cells through an NF- κ B-mediated increase in Nox4 expression and NADPH oxidase activity (Burdak-Rothkamm et al., 2007, 2008; Shao et al., 2008). Finally, the stimulation of the IL1R/NF- κ B pathway known to activate cellular inflammatory responses also cooperates with TGF β /SMAD to induce bystander senescence (Hubackova et al., 2012).

Conclusion and Perspectives

It is now clear that DNA-damaged cells interact with the extracellular environment to induce bi-directional changes within themselves and in undamaged neighboring cells. These communication strategies have most likely evolved to convey stress signals from damaged cells to the surrounding tissue and occur relatively rapidly (within hours) and/or slowly under the shape of the SASP. In the case of cancer treatment, therapeutic tools, including radiation and cytotoxic drugs, can trigger DDR activity and cellular senescence in normal and neoplastic cells but whether the generation of a DDR-driven immunomodulatory microenvironment has beneficial or detrimental consequences remains unknown (Acosta and Gil, 2012; Sun and Nelson, 2012). It is thus evident

that understanding microenvironment-modulating DDR-related mechanisms and their consequences remains a major challenge in the development of successful cancer therapies. Recent tools have emerged to directly manipulate senescence in mammalian model systems, which will be very useful in determining the importance of extracellular signals emitted from senescent cells (Baker et al., 2011; Laberge et al., 2013; Demaria et al., 2014). The use of these models and other strategies will be instrumental in the exploration of the pathways regulating DDR-mediated extracellular communication, as well as in the identification of extracellular signaling molecules that may become potential targets for therapeutic development in advanced

cancer therapies that take into account tissue microenvironments.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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